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FOLIAR PENETRATION OF A FUNGICIDE AS DETECTED BY SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS

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Abstract

Cu II hydroxide, $\text{Cu}(\text{OH})_2$ a fungicide, produces injury as a result of "chemical burn" or phytotoxicity on some plants. Scanning electron microscopy and energy dispersive X-ray analysis were used to characterize chlorosis on red maple leaves following foliar applications of $\text{Cu}(\text{OH})_2$. Copper was located with X-ray mapping in foliar mesophyll cells of trees sprayed with $\text{Cu}(\text{OH})_2$ but not in leaves of trees sprayed with distilled H_2O .

Introduction

Phytotoxicity or injury often accompanies the application of pesticides to plants. These injuries are known to vary in form and degree and are influenced by the developmental stage of the target plant, the species or cultivar, and by other environmental conditions (8). Some pesticides may affect photosynthesis and respiration and may frequently reduce fruit production and quality (1).

Injury from pesticides is difficult to diagnose accurately and usually requires more than symptom recognition on injured plants. Knowledge of site situations, growth conditions, previous pesticide applications, amounts and conditions under which they were applied should assist diagnosis (8). Many pesticides induce injury to plants and cause symptoms that mimic infectious diseases such as those caused by fungi, bacteria, viruses (8) or other abiotic stresses (2).

Cupric hydroxide, $\text{Cu}(\text{OH})_2$, is a fungicide commonly used to control fungal and bacterial foliar diseases (10). Phytotoxicity, in the form of marginal necrosis, is the result of $\text{Cu}(\text{OH})_2$ treatment on several nursery crops (7). Ayer and Barden (1) found that $\text{Cu}(\text{OH})_2$ can limit net photosynthesis of apple leaves. Cu interferes with protein synthesis (1), causes an increase in soluble nitrogen compounds (3) and has been found to attack phosphatases in *Agrostis tenuis* (9). Conventional techniques using light microscopic examination or standard chromatograph procedures are usually ineffective diagnostic methods (11). The purpose of this paper is to demonstrate an objective method for detecting phytotoxicity induced by $\text{Cu}(\text{OH})_2$.

Materials and Methods

Plant Material

Twenty 1-year-old *Acer rubrum* L. "Scarlet Sentinel" ramets were potted in a soil-peat-perlite (2:2:1, v/v/v) mixture in 25 cm diameter plastic pots and grown in a greenhouse. All plants were watered as needed and fertilized biweekly with nitrogen/phosphorus/potassium in a ratio of 20/8.6/16.6. Moisture and light conditions were not monitored.

Spray Treatment

Ten control trees were sprayed 6 times with double deionized H_2O at 14 day intervals. $\text{Cu}(\text{OH})_2$, applied as Kocide 101, 50W (Kocide Copper Co.) was sprayed 6 times on the ten other trees at the rate of 1.84 g/l. Fungicide treatments were applied to runoff with a pressurized sprayer operated at 2.1 kg/cm² (30 psi).

Electron Microscopy

Mature leaves with a plastochron leaf age of 10 (6) were

Keywords: Phytotoxicity, diagnosis, plant stress, abiotic disease, cupric hydroxide, fungicide distribution.

sampled 18 hours after each treatment for analysis by scanning electron microscopy (SEM) and energy dispersive x-ray analysis (EDX). Twenty fresh, hydrated and dehydrated leaf specimens from each treatment were mounted on carbon planchets with graphite adhesive and attached to aluminum stubs. Cross-sections were obtained by cutting the leaves with razor blades. To determine if beam and vacuum-induced artifacts were present in hydrated specimens, similar samples were fixed, dehydrated and critically-point-dried according to Krause (5) and mounted on carbon planchets. All samples were placed on a rotating, tilting stage within a vacuum evaporator and coated with carbon. They were subsequently examined with a Hitachi S-500 SEM equipped with a cold stage (@-180°C) to reduce heat artifacts during examination. The SEM was also equipped with a Tracor Northern-2000 EDX, employing a semi-quantitative identification program, a continuum subtraction program and digital beam control hardware with X-ray mapping programs.

The SEM was operated at 25 kV, tilt angle of 30°, take off angle of 61°, 400 sec of exposure and mapping at 0.01 sec/point using the x-ray display program from the manufacturer. Specimen preparation procedures are outlined in Figure 1.

Results and Discussion

Red maple leaves sprayed with H₂O did not exhibit lesions or necrosis but appeared turgid and vigorous as shown in Figure 2 (top leaf). For brevity and a reduction in the number of figures either micrographs of unfixed, hydrated specimens or micrographs of fixed, dehydrated specimens, (but not both) are used to describe the results of this experiment. Minor problems of surface structure collapse occurred in unfixed, hydrated specimens. When examined with SEM, top hydrated leaf surfaces had smooth epicuticular wax (Figure 3) and were similar to those described by Krause (4). Bottom hydrated surfaces (Figure 4) sprayed with H₂O had downy-like epicuticular wax with turgid, convex epidermal cells. EDX analysis revealed occasional particles containing silicon (Si) and calcium (Ca). Figure 5, a cross-section from a dehydrated leaf, showed various cell types as noted by Krause (5). Figure 6 shows a typical EDX spectrum from an *A. rubrum* leaf that was sprayed with H₂O. Endogenous elements were present as follows: Mg, Si, P, S, Cl, K, Ca and Fe. There were no obvious differences between the elemental composition of various cell types in the leaf.

Marginal necrosis began to be visible after two weeks on leaves sprayed with Cu(OH)₂, as shown at the bottom of Figure 2. Particles of Cu detected with SEM and EDX (Figures 7-15) on upper hydrated surfaces were uniformly distributed on leaves sprayed with Cu(OH)₂. Crystals were dispersed as shown in Figure 7. EDX indicated that Cu was present in the crystals as noted by the typical spectrum in Figure 8. X-ray dot mapping (Figure 9) verified the presence of Cu in the crystals. Aggregated crystals of Cu were visualized on the lower surfaces of leaves (see arrows in Figure 10) sprayed with Cu(OH)₂. Closer observation (Figure 11) indicated that these particles were loosely arranged and did not appear to be single, large crystals, but many smaller particles. Cu was detected in particles by EDX dot mapping (Figure 12). The variation in distribution of Cu between upper and lower surfaces and the difference in the texture of the epicuticular wax configuration are noteworthy. Perhaps the smooth upper leaf surface is more hydrophilic than the rough lower surface.

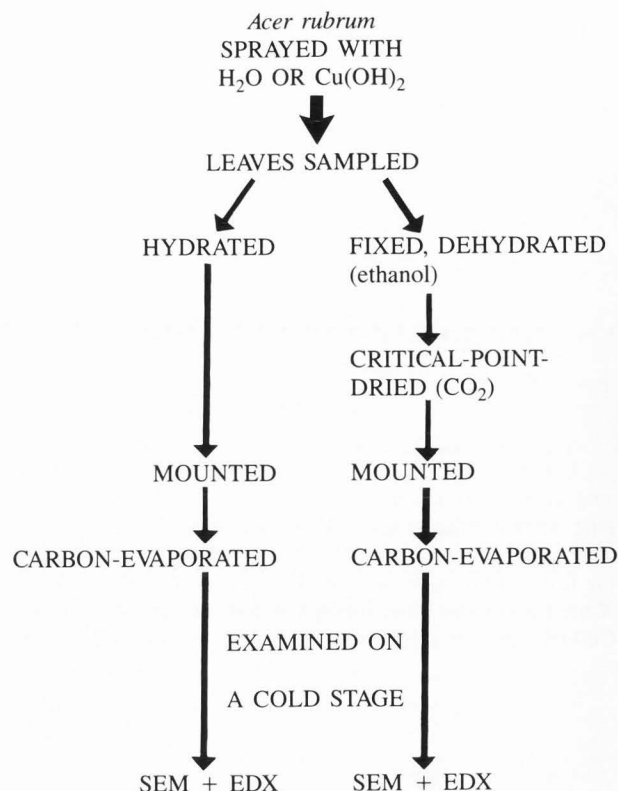


Figure 1. Specimen preparation procedures used in this study.

When 20 dehydrated leaf cross-sections from areas of marginal necrosis were examined with SEM (Figure 13), Cu was detected in mesophyll cells in all sections as noted by a typical spectrum (Figure 14) and X-ray dot mapping (Figure 15). X-ray analysis of non-necrotic areas failed to show the presence of Cu.

Cu toxicity expressed as marginal chlorosis appears to be related to the presence of Cu as detected by EDX. This is the first study to relate macroscopic symptoms with the presence of phytotoxins, namely Cu, and to visualize the agent in particular cell types, namely leaf mesophyll cells. Future studies will attempt to define the mode of action of Cu toxicity. Further definition of the role of Cu toxicity to specific organelles can be accomplished with scanning transmission electron microscopy on sections of affected leaf tissue.

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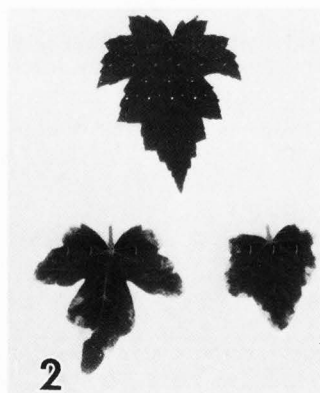


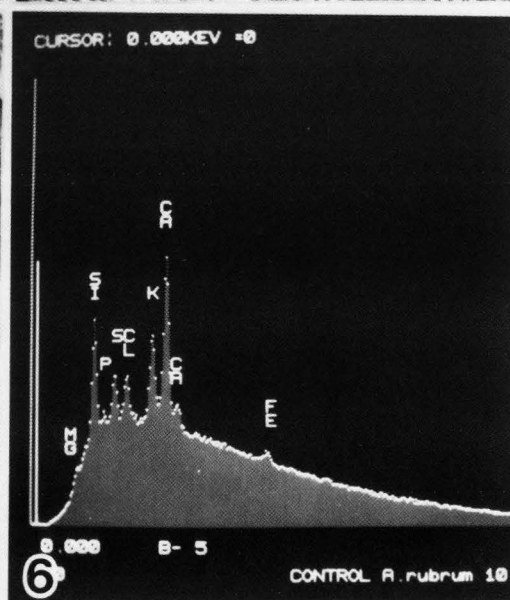
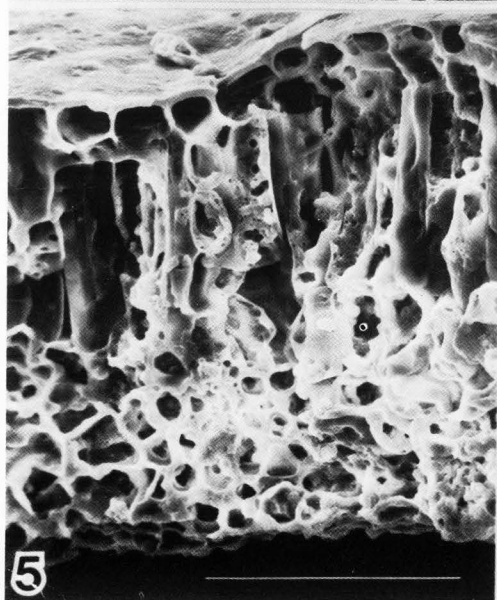
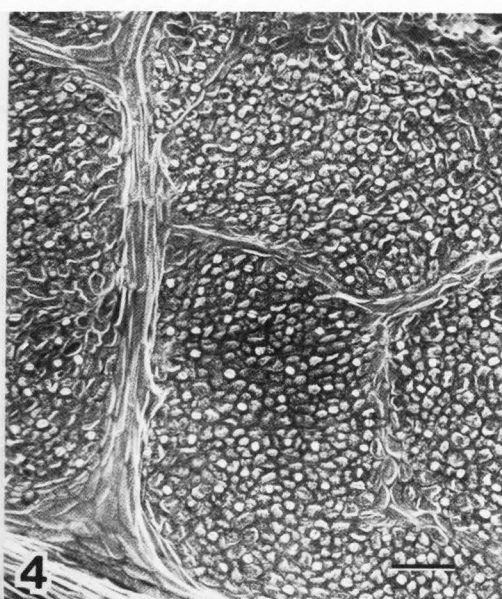
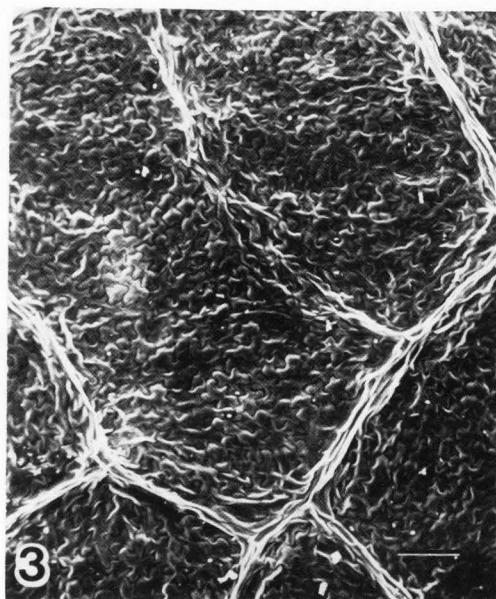
Figure 2. Top, red maple leaf sprayed with H₂O. Bottom, leaves sprayed with Cu(OH)₂ showing marginal necrosis.

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Figures 3-5. Scanning electron micrographs of unfixed, hydrated red maple leaves sprayed with H₂O. Figure 3. Upper surface with smooth epicuticular wax (bar=50μm). Figure 4. Bottom surface with downy-like epicuticular wax (bar =50μm). Figure 5. Cross-section showing various cell types (bar=50μm). Figure 6. Typical spectrum from H₂O-sprayed *A. rubrum*, leaf showing the endogenous elements Mg, Si, P, S, Cl, K, Ca and Fe.



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Discussion with Reviewers

A.L. Granett: What differences did you find in leaves examined at different times after spraying?

Author: Injury was expressed as marginal necrosis two weeks after the first spraying and symptoms became progressively more severe.

A.L. Granett: Were there any notable surface differences in Cu distribution between necrotic and non-necrotic areas?

Author: Copper crystals were evenly distributed on the surfaces of necrotic and non-necrotic areas. I feel that necrosis was related to the Cu penetration into mesophyll cells.

A.L. Granett: Would Cu distribution be affected if a wetting agent was present in the spray?

Author: I repeated the experiment with a wetting agent and did not observe any significant differences in Cu distribution.

A.L. Granett: Was any Cu detected in and around stomata?

Author: Cu crystals were not concentrated around guard cells even though some Cu appeared to be randomly distributed near stomata.

R.H. Falk: Is the author aware of the profound difficulties associated with X-ray microanalysis studies of conventionally prepared tissue and frozen hydrated tissues?

Author: Yes, I am aware that artifacts can creep into the analysis described in this paper. However, in this study, I emphasized two preparation techniques that corroborated the results. Other cryogenic methods (i.e., freeze substitution, freeze drying, etc.) that would further substantiate my results were not available. In research we all must work under some constraints.

L.W. Kress: Did leaves sampled near the end of the experiment receive multiple sprayings or were they leaves that developed later in the experiment?

Author: Leaves sampled and analyzed in the experiment received several sprayings of $\text{Cu}(\text{OH})_2$.

L.W. Kress: Was the amount of Cu detected inside the leaf related to the number of sprayings and was it quantified?

Author: I didn't attempt to correlate the number of sprayings with the amount of Cu detected inside the leaf.

Figures 7-15. Red maple leaves sprayed with $\text{Cu}(\text{OH})_2$. **Figure 7.** Particles detected on upper surface, note uniform distribution. **Figure 8.** An energy dispersive X-ray (EDX) spectrum of the hydrated upper surfaces of the specimen of Figure 7 indicating the presence of Cu and other elements. **Figure 9.** X-ray dot map of Figure 7 showing actual Cu distribution in the particles.

Figures 10,11. Lower hydrated surface showing a crystal identified as Cu with EDX.

Figure 12. X-ray dot map of Cu present in the particle of Figure 11.

Figure 13. Cross-section from areas of marginal necrosis obtained with a razor blade. **Figure 14.** EDX spectrum of cross section of Figure 13 (note predominance of Cu). **Figure 15.** X-ray dot map of Cu present in cross-section of leaf that exhibited marginal necrosis (Figure 13). **Figures 7 and 10,** bars= $50\mu\text{m}$. **Figures 11 and 13,** bars= $5\mu\text{m}$.

A.L. Granett: Could the differences in Cu distribution between upper and lower leaf surfaces depend on the method of spraying or gravitational forces?

Author: I sprayed plants "to runoff" so I don't feel the spray method influenced particle size or deposition. Since a separate experiment wasn't performed to test gravitational forces, I don't know if it was a significant factor.

G.M. Roomans: Were hydrated specimens slowly cooled on the stage or were they rapidly frozen before placing them on the stage?

Author: Hydrated specimens were slowly cooled without ice crystal formation but with occasional charging.

G.M. Roomans: Didn't the extremely rough surfaces of leaf cross-sections create interpretation problems in terms of X-ray absorption and take-off angles?

Author: Since irregular surfaces can induce artifacts into EDX studies, I have performed additional tests to resolve this question. First, count rates stayed approximately the same as I took spectra at decreasing magnifications (10,000, 8,000, 6,000) of the same rastered areas. This data indicates that the dark areas in the X-ray digital map were valid and void of characteristic Cu X-rays. Second, using the same rastered area the sample was rotated 5° and 10° without appreciable increase in counts.

D.M.R. Harvey: What are the possibilities of surface particle redistribution during preparation?

Author: Surface particle redistribution was a definite concern and was the primary reason that I used unfixed, hydrated samples for particle deposition analysis.

G.M. Roomans: How do you know that you only analyzed mesophyll cells for Cu in terms of spatial resolution?

Author: While X-ray spatial resolution wasn't determined in this study, I feel confident that only mesophyll cells contained Cu, since analysis of vascular and epidermal tissue didn't yield Cu.

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